COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCER, SCREENING OF PUTATIVE ANTI-CANCER COMPOUNDS, AND ASSESSING CANCER PROGRESSION

This invention claims priority under 35 U.S.C. §119(e) to US Provisional Application Number 60/427,326 filed November 18, 2002. The entire disclosure of the above-identified application is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the fields of cellular biology and oncology. Specifically, the present invention provides novel methods for the treatment of cancer, methods for screening compounds having anti-cancer activity, and methods of assessing cancer progression.

BACKGROUND OF THE INVENTION

A number of literature and patent references are cited in the instant application in order to more fully describe the state of the art to which this invention pertains. The entire disclosure of each of these citations is incorporated by reference herein.

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal malignancies of the GI tract. The neoplastic cells in GISTs appear to belong to the same lineage as the interstitial cells of Cajal (ICC), which are the pacemaker cells regulating gastrointestinal peristaltic activity. GISTs share many immunohistochemical, morphological, and ultrastructural

features with ICCs that support this hypothesis. In a
small number of cases, however, these tumors can be
located outside the GI tract (omentum, peritoneum, and
retroperitoneum) in tissues that are not known to contain
5   ICCs and, therefore, the true progenitor cell is
controversial. These GI sarcomas are characterized by
the presence of constitutively activated KIT (CD117), the
receptor tyrosine kinase (RTK) encoded by the c-KIT
proto-oncogene, also known as stem cell factor receptor.
10   c-KIT is a member of the RTK subclass III family and has
structural homology to the receptors for FLT3, platelet-
derived growth factor (PDGF) and macrophage colony-
stimulating factor. The proposed mechanism of
constitutive KIT tyrosine phosphorylation in most GIST is
15   a gain of function mutation in the c-KIT gene. These
mutations generally involve either missense nucleotide
substitutions or in-frame deletions, but certain GISTs
have in-frame insertions, resulting in c-KIT activation.
Most c-KIT mutations, in GISTs, involve exon 11, which
20   encodes a portion of the cytoplasmic juxtamembrane
domain. Smaller numbers of mutations involve, without
limitation, exon 9 (extracellular domain), exon 13 (first
part of the split tyrosine kinase domain), or exon 17
(phosphotransferase domain) [Lasota et al., Am. J.
25   Pathol. 154, 53-60 (1999); Rubin et al., Cancer Res. 61,
8118-8121 (2001); Hirota et al., Science 279, 577-580
(1998); Nakahara et al., Gastroenterology 115, 1090-1095
(1998); Lux et al., Am. J. Pathol. 156, 791-795 (2000)].
      Most GISTs are diagnosed in middle-aged and older
30   adults and can occur anywhere in the gastrointestinal
tract but predominantly are localized to the stomach.
Malignant GISTs often present with synchronous metastatic
disease and even those amenable to complete surgical

resection often recur locally, with a 5-year actuarial
survival rate of approximately 50%. Patients with
metastatic disease uniformly have a poor prognosis with a
median survival of 6-8 months, as GISTs have been
5 historically resistant to conventional and
investigational therapy.

Imatinib (imatinib mesylate, GLIVEC®/GLEEVEC®,
Novartis, Basel, Switzerland), formerly known as STI571,
is a 2-phenylaminopyrimidine derivative orally
10 administrated drug which in pre-clinical studies inhibits
c-Abl, Bcr-Abl, and PDGF receptors [Buchdunger et al., J.
Pharmacol. Exp. Ther. 295, 139-145 (2000); Druker, B.J.,
Trends Mol Med 8, S14-8. (2002)]. Imatinib (N-[4-methyl-
3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-4-(4-
15 methyl-piperazin-1-ylmethyl)-benzamide) is described,
e.g., in EP 0 564 409 B1 and, in the form of the methane
sulfonate salt, in WO 99/03854. Several phase I/II
clinical trials have demonstrated the efficacy of
imatinib in the treatment of CML patients with the
20 Philadelphia chromosome and the BCR/ABL translocation
[Kantarjian et al., N. Engl. J. Med. 346, 645-652 (2002);
Talpaz et al., Blood 98, 845a (2001); Sawyers et al.,
Blood 99, 3530-9. (2002)]. This drug has also been shown
to be a specific *in vitro* inhibitor of c-KIT
25 phosphorylation in several tumor cell lines [Buchdunger
et al., J. Pharmacol. Exp. Ther. 295, 139-145 (2000);
Heinrich et al., Blood 96, 925-932 (2000)]. After
suggestive initial pre-clinical studies it was proposed
that activated c-KIT was an early event in the initiation
30 and progression of the malignant phenotype for GIST. A
recent report indicated that imatinib rapidly inhibits c-
KIT phosphorylation and tumor cell proliferation, while
inducing apoptosis, in an established human GIST cell

line (GIST882). GIST882 is an immortal GIST cell line
that expresses an activating c-KIT mutation encoded by a
homozygous exon 13 missense mutation (i.e., K642E amino
acid substitution) [Tuveson et al., Oncogene 20, 5054-
5 5058 (2001)]. The KIT RTK target has been exploited in
two recently completed phase I/II clinical trials of
GIST. Over 50% of patients with unresectable or
metastatic GISTs demonstrated a classic partial response
and only 10% of patients manifested disease progression
10 while receiving imatinib [Blanke et al., Prog. Proc. Am.
Soc. Clin. Oncol. 21, 1608a (2002); van Oosterom et al.,
Lancet 358, 1421-1423 (2001)].

       Despite its early clinical success there are
limited pre-clinical and clinical data on the molecular
15 targets of imatinib downstream from c-KIT. In particular
there has not been a comprehensive evaluation of changes
in GIST gene expression following imatinib therapy.

SUMMARY OF THE INVENTION

20   In accordance with the present invention, a
method of assessing the anti-cancer activity of a KIT
tyrosine kinase inhibitor in a biological sample
comprising a tumor cell is provided. An exemplary method
comprises detecting alterations in expression levels of a
25 nucleic acid sequence provided in Tables 2, 3 and 4 in
response to administration the KIT tyrosine kinase
inhibitor. The present inventors have identified
specific alterations in expression patterns of the
sequences listed in Tables 2, 3 and 4 in response to
30 administration of anti-cancer agents. In a preferred
embodiment, the tumor is a gastrointestinal stromal tumor
and the KIT tyrosine kinase inhibitor is imatinib,
SU11248 (Sugen Pharmaceuticals), or a pharmaceutically

acceptable salt thereof. In a particularly preferred
embodiment, the method comprises detecting the level of
expression of a nucleic acid sequence encoding Sprouty4A
protein. The method also encompasses determining

5   expression levels of the Sprouty4A protein itself using
an antibody that specifically binds to the Sprouty4A
protein.

Also within the scope of the present invention is a
method of screening a compound for activity against a

10   tumor. An exemplary method comprises contacting
mammalian tumor cells with the compound and detecting in
said tumor cells an altered level of expression of a
nucleic acid listed in at least one table selected from
the group consisting of Table 2, Table 3 and Table 4

15   relative to a control. In a preferred embodiment,
compounds are screened using the gastrointestinal stromal
tumor cell line, GIST882.

In yet another aspect, a method for determining the
efficacy of an anticancer treatment comprising detection

20   of an alteration in expression levels of a biomarker are
provided. Suitable biomarkers for this purpose comprise
a nucleic sequence provided in a table selected from the
group consisting of Table 2, Table 3, and Table 4.
Preferably, the biomarker is sprouty 4, the tumor is GIST

25   and sprouty 4 expression level is decreased in response
to effective anticancer treatment. In an alternative
embodiment, the tumor is GIST, the biomarker is MAFbx and
MAFbx expression level is elevated in response to
effective anticancer treatment.

30   In yet another aspect of the invention, a method for
determining the efficacy of an anticancer treatment
comprising detection of an alteration in post-translation
modification of a biomarker is provided. Again, suitable

nucleic acids for this purpose comprise a nucleic
sequence provided in a table selected from the group
consisting of Table 2, Table 3, and Table 4. In a
preferred embodiment, the tumor is GIST, the biomarker is
5   GAB1, and the post translational modification is
phosphorylation which is decreased in response to
effective anti-cancer treatment.

BRIEF DESCRIPTIONS OF THE DRAWING

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Figure 1 is an image of a cDNA microarray analysis of
SPRY4A (Sprouty4A) expression. Snapshots of actual
hybridization signal pairs from spotted SPRY4A cDNA
fragment when hybridized with Cy3- and Cy5- labeled cDNA
15   from GIST cells treated and untreated with 10 μM imatinib
for 6, 12, 24, and 48 hours. The last pair (C)
represents hybridization signals of β-actin in both
channels as a control.

20   Figure 2A is a graph of a growth curve of imatinib-
treated and untreated control cells. The X axis
represents hours of treatment and the Y axis represents
the total number of cells (X10^5). Untreated GIST882 cells
(diamonds); GIST882 cells treated with 1 μM imatinib
25   (squares); GIST882 cells treated with 10 μM imatinib
(triangles). Figure 2B is a schematic alignment of
SPRY4A [GenBank Accession No. AF227516 (Homo sapiens
Sprouty4A mRNA, complete cDNA); Protein ID: AAK00652.1]
and SPRY4C [GenBank Accession No. AF227517 (Homo sapiens
30   Sprouty4C mRNA, complete cDNA); Protein ID: AAK00653.1].
Diagonally shaded areas represent sequence unique for
SPRY4A, vertically shaded area is the sequence unique for
SPRY4C, black box represents the fragment of the cDNA

sequence spotted on the microarray, arrows indicate the
position of primer sets used for reverse transcription
polymerase chain reaction (RT-PCR) analysis. Figure 2C
is an image of a Northern blot analysis of SPRY4A

5   expression. In the upper panel, the SPRY4A mRNA levels
in imatinib treated and control GIST cells are shown. In
the lower panel, an image of the ethidium bromide-stained
gel prior to blotting is shown. The position of the 28s
and 18s rRNA is indicated at the left of the panels. ·

10   Figure 2D is an image of a gel representing the RT-PCR
analysis of SPRY4 expression. In the upper panel, the
isoform determination and the level of the SPRY4 RT-PCR
product in imatinib treated and untreated GIST cells are
shown. In the lower panel, β-actin RT-PCR product levels

15   in drug treated and untreated GIST cells are shown.

Figure 3A is a series of immunoblots showing the levels
of phospho-c-Kit/total c-Kit, phospho-AKT/total AKT, and
phospho-ERK1/2/total ERK1/2 in imatinib (1 and 10 μM)

20   treated and untreated GIST cells. Figure 3B is an image
of an RT-PCR analysis of SPRY4A and β-actin levels in
imatinib treated and untreated GIST cells.

Figure 4A is a series of immunoblots showing phospho-
25   ERK1/2/total ERK1/2, phospho-AKT/total AKT levels in
U0126, a MEK inhibitor, treated and untreated GIST cells.
Figure 4B is an image of an RT-PCR analysis of SPRY4A and
β-actin levels in U0126 treated and untreated GIST cells.

30   Figure 5A is a gel image showing SPRY4A expression in
response to imatinib treatment in clinical cases of GIST.
In the upper panel, the levels of SPRY4A RT-PCR product
is shown before and after drug administration in six

patients with GIST [patients #1, 2, 3 responded favorably
to the drug, patient #4 initially responded but
subsequently progressed (R-P), patients #5 and 6 failed
to respond to treatment (NR), patient #7 had one tumor

5   respond to treatment and one tumor that did not]. In the
lower panel, β-actin RT-PCR product levels are shown as a
control. Figure 5B is a gel image showing the RT-PCR
analysis of MAFbx for patients 1, 4, 6, and 7. The lower
panel is a β-actin control.

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Figure 6 is a series of immunoblots showing the phospho-
c-KIT and total c-KIT protein (Fig. 6A); phospho-AKT and
total AKT levels (Fig. 6B); and phospho-ERK1/2 and total
ERK1/2 levels (Fig. 6C) in liposarcoma, GIST cells and

15 imatinib resistant GIST.

Figure 7A is a series of gel images showing the RT-PCR
analysis of SPRY1, 2, 3, and 4 in untreated and imatinib
treated GIST cells. β-actin is provided as a control.

20 Figure 7B is a Western blot, employing a SPRY4 specific
antibody, of lysates from GIST cells treated with
imatinib for 0, 24, 72, and 120 hours.

Figure 8A is a series of gel images of the RT-PCR

25 analysis of imatinib-responsive genes. RNA was isolated
from imatinib-treated (10 μM for 6, 12, 24, and 48 h) and
untreated GIST882 cells and reverse transcribed. Shown
are ethidium bromide stained agarose gels of the RT-PCR
products for SPRY4A, MAFbx, FZD8, PDE2A, RTP801,

30 FLJ20898, and ARHGEF2. β-Actin is shown as a control.
Fig. 8B is a series of gel images of the RT-PCR analysis
of SPRY4A, MAFbx, FZD8, PDE2A, RTP801, FLJ20898, ARHGEF2,
and β-actin levels in U0126-treated and untreated GIST

cells. Fig. 8C a series of gel images of the RT-PCR
analysisof SPRY4A, MAFbx, FZD8, PDE2A, RTP801, FLJ20898,
ARHGEF2, and β-actin levels in imatinib-treated and
untreated GIST cells. Fig. 8D is a series of gel images
5 of the RT-PCR analysis of SPRY4A, MAFbx, and β-actin
levels in LY294002-treated and untreated GIST cells.

Figure 9 is a series of Western blots. GIST cells were
incubated with imatinib mesylate for 5, 15, 30, 60, and
10 120 minutes (lanes 1-6, respectively). The cells were
lysed and proteins were separated by acrylamide gel
electrophoresis and Western blotting was performed using
antibodies to GAB1 and phospho-GAB1 (P-GAB1; upper panel)
or GAB2 and phosph-GAB2 (P-GAB2; lower panel).
15 Antibodies were obtained from Cell Signaling Technology
with the exception of GAB2 which was obtained from
Upstate Biotechnology.

DETAILED DESCRIPTION OF THE INVENTION

20   DNA microarrays revealed 148 genes that were
differentially expressed between untreated and imatinib-
treated human GIST cells, *in vitro*. One of these genes,
Sprouty4A (SPRY4A) a regulator of tyrosine kinase-
mediated signaling pathways, was dramatically down-
25 regulated (~7-fold) for the entire duration of drug
exposure (6-48 h). In addition, imatinib inhibited KIT
phosphorylation without affecting the total level of KIT
protein. The constitutive activation of extra-cellular
signal regulated kinase (ERK) 1/2 and AKT was also
30 inhibited without affecting the total cellular levels of
either protein, however, down-regulation of SPRY4A was
dependent on the activation of the ERK1/2 pathway and was
independent of AKT activation. To investigate the

clinical correlation of these findings GIST specimens
were examined from patients on a phase II imatinib
treatment trial. Tumor biopsies from patients with non-
responding GIST continued to express high levels of
5   SPRY4A, whereas SPRY4A expression was down-regulated in
GIST biopsies obtained during imatinib therapy from
patients who clinically demonstrated durable partial
response. Evaluation of available tissue from one of the
non-responder found that high levels of SPRY4A mRNA
10   positively correlated with expression of constitutively
active forms of c-KIT and ERK1/2. Furthermore, this
tumor possessed a mutation in exon 9 of c-KIT that was
associated with a failure to respond to imatinib. This
in frame mutation (1530ins6) in exon 9 of c-KIT has
15   previously been described in a study of Japanese GIST
patients [Sakurai et al., Jpn J Cancer Res 92, 494-8
(2001)], but has not been correlated with resistance to
imatinib.
         Sprouty was originally identified as a down-
20   regulator of the "Breathless" (the Drosophila equivalent
of fibroblast growth factor receptor) signaling cascade
that governs tracheal branching [Hacohen et al., Cell 92,
253-63 (1998)]. Sprouty is an intracellular protein
associated with the inner surface of the plasma membrane
25   that binds two intracellular components of the Ras
pathway, Drk (the Drosophila homolog of Grb2) and Gap1, a
Ras GTPase-activating protein [Casci et al., Cell 96,
655-65 (1999)]. Sprouty is an inhibitor of Ras signal
transduction in particular and of RTK signaling in
30   general [Casci et al., Cell 96, 655-65 (1999); Reich et
al., Development 126, 4139-47. (1999)]. While Drosophila
has only one Sprouty protein, mammals have at least five
related proteins (Sprouty 1, 2, 3, 4A, and 4C) encoded by

four genes. The Sprouty proteins are classified under
the same gene family by virtue of their characteristic
cysteine-rich residues located in their carboxyl termini.

5   SPRY4A (sometimes referred to herein as SPRY4) may
also have an active role in melanocytes, mastocytes and
other c-KIT expressing cells. SPRY4A is constitutively
expressed in liposarcomas and leiomiosarcomas (data not
shown), sarcoma histotypes that as a group are
unresponsive to imatinib. In this respect, it has been
10   shown that c-KIT is not expressed in these tumors (Fig. 6
and data not shown), but that ERK1/2 is constitutively
activated. Therefore, it is possible that SPRY4A
expression in these tumors may be regulated via the EGFR
signaling pathway given that EGF treatment can also
15   induce Spry4 expression in mammalian cells [Sasaki et
al., J Biol Chem 276, 36804-8 (2001)]. Sprouty has also
been demonstrated to interact with Grb2 [Casci et al.,
Cell 96, 655-65 (1999)] and Grb2, in turn, has been found
to be tyrosine phosphorylated by Bcr/Abl [Li et al., Embo
20   J 20, 6793-804 (2001)]. These findings further implicate
Sprouty in the therapeutic response to imatinib seen
among CML patients.

As demonstrated herein, expression of SPRY4A is a
reliable marker of KIT tyrosine kinase inhibitor response
25   in GISTs. The findings also point to SPRY4A as a
potential genetic endpoint of the KIT tyrosine kinase
inhibitor-induced arrest of the c-KIT signaling pathway.
This can translate into the clinical application of
SPRY4A expression as a tool for early discrimination
30   between patients that respond to therapy with a KIT
tyrosine kinase inhibitor such as imatinib and those who
will progress.

The level of expression of a gene whose expression level correlates with the biological activity of a KIT tyrosine kinase inhibitor can be measured by any technical means such as, without limitation, by

5   monitoring RNA expression by using, for example, the techniques of RT-PCR or Northern blotting; or by monitoring protein expression by using, for example, the techniques of Western blotting, ELISA or immunohistochemistry.

10   Antibodies that specifically bind to the Sprouty4A protein can be produced in accordance with known methods. The Sprouty4A protein or parts thereof can be produced, e.g., by conventional recombinant DNA techniques or, alternatively, be synthesized chemically using standard

15   peptide synthesis techniques. These proteins/peptides can then be used to generate antibodies directed against the Sprouty4A protein using known immunization methods. In order to generate antibodies which bind to Sprouty4A but not to Sprouty4C or other Sprouty proteins, the

20   antibodies are preferably raised against peptide sequences which are present in the Sprouty4A isoform but not in the Sprouty4C isoform or other Sprouty proteins, such as, for example, the N-terminal sequence which has minimal homology among the Sprouty family. Antibodies

25   that specifically bind to the Sprouty4A protein can be used to detect the level of expression of the Sprouty4A protein in a biological sample by means of, e.g., Western blotting, ELISA, or immunohistochemistry.

    The general terms used hereinbefore and hereinafter

30   preferably have the following meanings, if not indicated otherwise:

    "Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single

or double stranded and, if single stranded, the molecule
of its complementary sequence in either linear or
circular form. In discussing nucleic acid molecules, a
sequence or structure of a particular nucleic acid
5   molecule may be described herein according to the normal
convention of providing the sequence in the 5' to 3'
direction. With reference to nucleic acids of the
invention, the term "isolated nucleic acid" is sometimes
used. This term, when applied to DNA, refers to a DNA
10 molecule that is separated from sequences with which it
is immediately contiguous in the naturally occurring
genome of the organism in which it originated. For
example, an "isolated nucleic acid" may comprise a DNA
molecule inserted into a vector, such as a plasmid or
15 virus vector, or integrated into the genomic DNA of a
prokaryotic or eukaryotic cell or host organism.

       When applied to RNA, the term "isolated nucleic
acid" refers primarily to an RNA molecule encoded by an
isolated DNA molecule as defined above. Alternatively,
20 the term may refer to an RNA molecule that has been
sufficiently separated from other nucleic acids with
which it would be associated in its natural state (i.e.,
in cells or tissues). An isolated nucleic acid (either
DNA or RNA) may further represent a molecule produced
25 directly by biological or synthetic means and separated
from other components present during its production.

       "Natural allelic variants", "mutants" and
"derivatives" of particular sequences of nucleic acids
refer to nucleic acid sequences that are closely related
30 to a particular sequence but which may possess, either
naturally or by design, changes in sequence or structure.
By closely related, it is meant that at least about 75%,
but often, more than 90%, of the nucleotides of the

sequence match over the defined length of the nucleic
acid sequence. Changes or differences in nucleotide
sequence between closely related nucleic acid sequences
may represent nucleotide changes in the sequence that
5   arise during the course of normal replication or
duplication in nature of the particular nucleic acid
sequence. Other changes may be specifically designed and
introduced into the sequence for specific purposes, such
as to change an amino acid codon or sequence in a
10   regulatory region of the nucleic acid. Such specific
changes may be made in vitro using a variety of
mutagenesis techniques or produced in a host organism
placed under particular selection conditions that induce
or select for the changes. Such sequence variants
15   generated specifically may be referred to as "mutants" or
"derivatives" of the original sequence.

The term "functional" as used herein implies that
the nucleic or amino acid sequence is functional for the
recited assay or purpose.

20   A "replicon" is any genetic element, for example, a
plasmid, cosmid, bacmid, phage or virus, that is capable
of replication largely under its own control. A replicon
may be either RNA or DNA and may be single or double
stranded.

25   A "vector" is a replicon, such as a plasmid, cosmid,
bacmid, phage or virus, to which another genetic sequence
or element (either DNA or RNA) may be attached so as to
bring about the replication of the attached sequence or
element.

30   An "expression operon" refers to a nucleic acid
segment that may possess transcriptional and
translational control sequences, such as promoters,
enhancers, translational start signals (e.g., ATG or AUG

codons), polyadenylation signals, terminators, and the
like, and which facilitate the expression of a
polypeptide coding sequence in a host cell or organism.

The term "probe" as used herein refers to an
5   oligonucleotide, polynucleotide or nucleic acid, either
RNA or DNA, whether occurring naturally as in a purified
restriction enzyme digest or produced synthetically,
which is capable of annealing with or specifically
hybridizing to a nucleic acid with sequences
10 complementary to the probe. A probe may be either
single-stranded or double-stranded. The exact length of
the probe will depend upon many factors, including
temperature, source of probe and use of the method. For
example, for diagnostic applications, depending on the
15 complexity of the target sequence, the oligonucleotide
probe typically contains 15-25 or more nucleotides,
although it may contain fewer nucleotides. The probes
herein are selected to be "substantially" complementary
to different strands of a particular target nucleic acid
20 sequence. This means that the probes must be
sufficiently complementary so as to be able to
"specifically hybridize" or anneal with their respective
target strands under a set of pre-determined conditions.
Therefore, the probe sequence need not reflect the exact
25 complementary sequence of the target. For example, a
non-complementary nucleotide fragment may be attached to
the 5' or 3' end of the probe, with the remainder of the
probe sequence being complementary to the target strand.
Alternatively, non-complementary bases or longer
30 sequences can be interspersed into the probe, provided
that the probe sequence has sufficient complementarity
with the sequence of the target nucleic acid to anneal
therewith specifically.

The term "specifically hybridize" refers to the
association between two single-stranded nucleic acid
molecules of sufficiently complementary sequence to
permit such hybridization under pre-determined conditions
5   generally used in the art (sometimes termed
"substantially complementary"). In particular, the term
refers to hybridization of an oligonucleotide with a
substantially complementary sequence contained within a
single-stranded DNA or RNA molecule of the invention, to
10 the substantial exclusion of hybridization of the
oligonucleotide with single-stranded nucleic acids of
non-complementary sequence.

In accordance with the present invention, nucleic
acids having the appropriate sequence homology with the
15 nucleic acids of the invention may be identified by using
hybridization and washing conditions of appropriate
stringency. For example, hybridizations may be performed,
according to the method of Sambrook et al. (1989,
Molecular Cloning, Cold Spring Harbor Laboratory), using
20 a hybridization solution comprising : 5X SSC, 5X
Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured,
fragmented salmon sperm DNA, 0.05% sodium pyrophosphate
and up to 50% formamide. Hybridization is carried out at
37-42°C for at least six hours. Following hybridization,
25 filters are washed as follows: (1) 5 minutes at room
temperature in 2X SSC and 1% SDS ; (2) 15 minutes at room
temperature in 2X SSC and 0.1% SDS ; (3) 30 minutes-1
hour at 37°C in 1X SSC and 1% SDS ; (4) 2 hours at 42-
65°C in 1X SSC and 1% SDS, changing the solution every 30
30 minutes.

One common formula for calculating the stringency
conditions required to achieve hybridization between

nucleic acid molecules of a specified sequence homology
(Sambrook et al., 1989, supra):

$$T_m = 81.5°C + 16.6 \text{ Log } [Na+] + 0.41 (\% \text{ G+C}) - 0.63 (\%$$
5   formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] =
0.368 and 50% formamide, with GC content of 42% and an
average probe size of 200 bases, the T_m is 57°C. The T_m of
10 a DNA duplex decreases by 1 - 1.5°C with every 1%
decrease in homology. Thus, targets with greater than
about 75% sequence identity would be observed using a
hybridization temperature of 42°C.
      The stringency of the hybridization and wash depend
15 primarily on the salt concentration and temperature of
the solutions. In general, to maximize the rate of
annealing of the probe with its target, the hybridization
is usually carried out at salt and temperature conditions
that are 20-25°C below the calculated T_m of the hybrid.
20 Wash conditions should be as stringent as possible for
the degree of identity of the probe for the target. In
general, wash conditions are selected to be approximately
12-20°C below the T_m of the hybrid. In regards to the
nucleic acids of the current invention, a moderate
25 stringency hybridization is defined as hybridization in
6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml
denatured salmon sperm DNA at 42°C, and washed in 2X SSC
and 0.5% SDS at 55°C for 15 minutes. A high stringency
hybridization is defined as hybridization in 6X SSC, 5X
30 Denhardt's solution, 0.5% SDS and 100 μg/ml denatured
salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5%
SDS at 65°C for 15 minutes. A very high stringency
hybridization is defined as hybridization in 6X SSC, 5X

Denhardt's solution, 0.5% SDS and 100 μg/ml denatured
salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5%
SDS at 65°C for 15 minutes.

The terms "transform", "transfect", "transduce",
5   shall refer to any method or means by which a nucleic
acid is introduced into a cell or host organism and may
be used interchangeably to convey the same meaning. Such
methods include, but are not limited to, transfection,
electroporation, microinjection, PEG-fusion and the like.
10   The introduced nucleic acid may or may not be integrated
(covalently linked) into nucleic acid of the recipient
cell or organism. In bacterial, yeast, plant and
mammalian cells, for example, the introduced nucleic acid
may be maintained as an episomal element or independent
15   replicon such as a plasmid. Alternatively, the introduced
nucleic acid may become integrated into the nucleic acid
of the recipient cell or organism and be stably
maintained in that cell or organism and further passed on
or inherited to progeny cells or organisms of the
20   recipient cell or organism. In other manners, the
introduced nucleic acid may exist in the recipient cell
or host organism only transiently.

A "cell line" is a clone of a primary cell or cell
population that is capable of stable growth in vitro for
25   many generations.

Suitable chemotherapeutic agents are any compounds
that exhibit anticancer activity including, but are not
limited to: alkylating agents (e.g., nitrogen mustards
such as chlorambucil, cyclophosphamide, isofamide,
30   mechlorethamine, melphalan, and uracil mustard;
aziridines such as thiotepa; methanesulphonate esters
such as busulfan; nitroso ureas such as carmustine,
lomustine, and streptozocin; platinum complexes such as

cisplatin and carboplatin; bioreductive alkylators such
as mitomycin, procarbazine, dacarbazine and altretamine);
DNA strand-breakage agents (e.g., bleomycin);
topoisomerase II inhibitors (e.g., amsacrine,

5   dactinomycin, daunorubicin, idarubicin, mitoxantrone,
doxorubicin, etoposide, and teniposide); DNA minor groove
binding agents (e.g., plicamydin); antimetabolites (e.g.,
folate antagonists such as methotrexate and trimetrexate;
pyrimidine antagonists such as fluorouracil,

10   fluorodeoxyuridine, CB3717, azacitidine, cytarabine, and
floxuridine; purine antagonists such as mercaptopurine,
6-thioguanine, fludarabine, pentostatin; asparginase; and
ribonucleotide reductase inhibitors such as hydroxyurea);
tubulin interactive agents (e.g., vincristine,

15   vinblastine, and paclitaxel (Taxol)); hormonal agents
(e.g., estrogens; conjugated estrogens; ethinyl
estradiol; diethylstilbesterol; chlortrianisen;
idenestrol; progestins such as hydroxyprogesterone
caproate, medroxyprogesterone, and megestrol; and

20   androgens such as testosterone, testosterone propionate,
fluoxymesterone, and methyltestosterone); adrenal
corticosteroids (e.g., prednisone, dexamethasone,
methylprednisolone, and prednisolone); leutinizing
hormone releasing agents or gonadotropin-releasing

25   hormone antagonists (e.g., leuprolide acetate and
goserelin acetate); and antihormonal antigens (e.g.,
tamoxifen, antiandrogen agents such as flutamide; and
antiadrenal agents such as mitotane and
aminoglutethimide). Preferably, the chemotheraputic

30   agent is selected from the group consisting of:
placitaxel (Taxol®), cisplatin, docetaxol, carboplatin,
vincristine, vinblastine, methotrexate, cyclophosphamide,
CPT-11, 5-fluorouracil (5-FU), gemcitabine, estramustine,

carmustine, adriamycin (doxorubicin), etoposide, arsenic
trioxide, irinotecan, and epothilone derivatives.

    The term "KIT tyrosine kinase inhibitor" is
understood to mean that in the tyrosine kinase inhibition
5 assay described below (c-Kit Enzyme Assay), such a
compound inhibits the tyrosine kinase activity of the c-
Kit tyrosine kinase domain by 50% (IC_{50}) in a
concentration of less than 10 μM, preferably less than 1
μM, more preferably less than 100 nM, most preferably
10 less than 10 nM. In a broader sense, the term "KIT
tyrosine kinase inhibitor" also refers to compounds which
decrease KIT tyrosine kinase activity indirectly, i.e.,
not through direct inhibition of the tyrosine kinase
domain of KIT. Such compounds may, for example, decrease
15 the expression levels of KIT, inhibit the pathway that
leads to activation of KIT or act as KIT antagonists.
Methods to test a compound's ability to exhibit such a
KIT inhibitory activity are well known in the art.
Preferably, the following is meant by a KIT tyrosine
20 kinase inhibitor: Imatinib, PTK787 [1-(4-chloroanilino)-
4-(4-pyridylmethyl)phthalazine; see US 6,258,812],
PD180970, ZD4190, SU5416, SU6668 and pharmaceutically
acceptable salts of these compounds, especially imatinib
and pharmaceutically acceptable salts thereof, most
25 preferably imatinib mesylate (GLIVEC®/GLEEVEC®).
Imatinib mesylate can be administered, e.g., in the form
as it is marketed under the trademark GLIVEC® or
GLEEVEC®.

    As an example of a c-Kit Enzyme Assay, the
30 baculovirus donor vector pFbacG01 (GIBCO) may be used to
generate a recombinant baculovirus that expresses the
amino acid region amino acids 544-976 of the cytoplasmic
kinase domain of human c-Kit. The coding sequences for

the cytoplasmic domain of c-Kit may be amplified by PCR from a human uterus c-DNA library (Clontech). The amplified DNA fragment and the pFbacG01 vector may be made compatible for ligation by digestion with BamHI and

5   EcoRI. Ligation of these DNA fragments results in the baculovirus donor plasmid c-Kit. The production of the viruses, the expression of proteins in Sf9 cells and the purification of the glutathione-S-transferase (GST)-fused proteins may be performed as follows:

10   *Production of virus*: Transfer vector (pFbacG01-c-Kit) containing the c-Kit kinase domain is transfected into the DH10Bac cell line (GIBCO) and the cells are plated on selective agar plates. Colonies without insertion of the fusion sequence into the viral genome

15   (carried by the bacteria) are blue. Single white colonies are picked and viral DNA (bacmid) is isolated from the bacteria by standard plasmid purification procedures. Sf9 or Sf21 cells (American Type Culture Collection) are then transfected in 25 cm^2 flasks with the

20   viral DNA using Cellfectin reagent.

Determination of small scale protein expression in Sf9 cells: Virus containing media is collected from the transfected cell culture and used for infection to increase its titer. Virus containing media obtained

25   after two rounds of infection is used for large-scale protein expression. For large-scale protein expression 100 cm^2 round tissue culture plates are seeded with 5 x 10^7 cells/plate and infected with 1 mL of virus-containing media (approx. 5 MOIs). After 3 days the cells are

30   scraped off the plate and centrifuged at 500 rpm for 5 min. Cell pellets from 10-20, 100 cm^2 plates, are resuspended in 50 mL of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM

PMSF). The cells are stirred on ice for 15 min and then centrifuged at 5000 rpms for 20 min.

Purification of GST-tagged protein: The centrifuged cell lysate is loaded onto a 2 mL glutathione-sepharose
5   column (Pharmacia) and washed three times with 10 mL of 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 200 mM NaCl. The GST-tagged protein is eluted by 10 applications (1 mL each) of 25 mM Tris-HCl, pH 7.5, 10 mM reduced-glutathione, 100 mM NaCl, 1 mM DTT, 10% Glycerol and
10 stored at -70°C.

Kinase assay: Tyrosine protein kinase assays with purified GST-c-Kit are carried out in a final volume of 30 µL containing 200-1800 ng of enzyme protein (depending on the specific activity), 20 mM Tris-HCl, pH 7.6, 3 mM
15 $MnCl_2$, 3 mM $MgCl_2$, 1 mM DTT, 10 µM Na_3VO_4, 5 µg/mL poly(Glu,Tyr) 4:1, 1% DMSO, 1.0 µM ATP and 0.1 µCi [γ^{33} P] ATP. The activity is assayed in the presence or absence of inhibitors, by measuring the incorporation of ^{33}P from [γ^{33} P] ATP into the poly(Glu,Tyr) substrate. The assay
20 (30 µL) is carried out in 96-well plates at ambient temperature for 20 min under conditions described below and terminated by the addition of 20 µL of 125 mM EDTA. Subsequently, 40 µL of the reaction mixture is transferred onto Immobilon-PVDF membrane (Millipore,
25 Bedford, MA, USA) previously soaked for 5 min with methanol, rinsed with water, then soaked for 5 min with 0.5% H_3PO_4 and mounted on vacuum manifold with disconnected vacuum source. After spotting all samples, vacuum is connected and each well rinsed with 200 µL 0.5%
30 H_3PO_4. Membranes are removed and washed 4 x on a shaker with 1.0% H_3PO_4 and once with ethanol. Membranes are counted after drying at ambient temperature, mounting in Packard TopCount 96-well frame, and addition of 10

μL/well of Microscint TM (Packard). IC_{50} values are
calculated by linear regression analysis of the
percentage inhibition of each compound in duplicate, at
four concentrations (usually 0.01, 0.1, 1 and 10 μM). One

5   unit of protein kinase activity is defined as 1 nmole of
^{33}P ATP transferred from $[\gamma^{33} P]$ ATP to the substrate
protein per minute per mg of protein at 37°C.

The structure of the active agents identified by
10 code nos., generic or trade names may be taken from the
actual edition of the standard compendium "The Merck
Index" or from databases, e.g., Patents International
(e.g. IMS World Publications). The corresponding content
thereof is hereby incorporated by reference.

15   Any reference hereinbefore and hereinafter to a free
KIT tyrosine kinase inhibitor is to be understood as
referring also to the corresponding pharmaceutically
acceptable salts thereof, as appropriate and expedient.
The term "a gene whose expression level correlates

20 with the biological activity of a KIT tyrosine kinase
inhibitor" is understood to mean that the level of
expression of such a gene in, e.g., a mammalian cell is
related to the extent of inhibition of the KIT tyrosine
kinase activity induced by an administered KIT tyrosine

25 kinase inhibitor. In a more specific sense, this term is
understood to mean that the level of expression of such a
gene is related to the anti-tumor activity, especially
anti-GIST activity, of an administered KIT tyrosine
kinase inhibitor. A gene whose expression level

30 correlates with the biological activity of a KIT tyrosine
kinase inhibitor is preferably a gene listed in the Table
below under Example 1, most preferably the Sprouty4A
gene.

The term "biological sample" is meant to include any biological material separated from the mammalian body such as, e.g., tissue, cell lines, plasma, serum, and the like.

5   Preferably human is meant by the term "mammal" or "mammalian".

The term "*ex vivo*" means outside the body of the mammal.

The term "antibody that specifically binds to the
10   Sprouty4A protein" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds to the Sprouty4A protein. A molecule which specifically binds to the
15   Sprouty4A protein is a molecule which binds to the Sprouty4A protein, but does not substantially bind to other molecules in a sample, e.g., a biological sample which naturally contains Sprouty4A. Preferably, antibodies that specifically bind to the Sprouty4A
20   protein do also not bind to the Sprouty4C isoform, i.e., such antibodies are isoform-specific in that they only recognize epitopes which are present in Sprouty4A but not in Sprouty4C. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and
25   F(ab')$_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The term "antibody" includes polyclonal and monoclonal antibodies, wherein the term "monoclonal antibody" refers to a population of antibody molecules that contain only one
30   species of an antigen binding site capable of immunoreacting with a particular epitope. Scfv produced by genetic engineering are also encompassed by the term antibody.

24

According to another aspect of the present
invention, the nucleic acid sequences identified as
differentially regulated in tumor cells treated with
5   imatinib can be employed to treat cancer (see Example 1,
Tables 2-4). Specifically, in cases where imatinib gives
rise to a beneficial therapeutic effect, nucleic acid
molecules, or fragments thereof, containing genes which
were upregulated after imatinib treatment may be
10   administered to cancer cells to increase expression and
thereby further inhibit tumor growth. Alterations in the
physiological amount of the genes may act synergistically
with chemotherapeutic agents used to treat cancer. In
one embodiment, the nucleic acid molecules of the
15   invention will be used to restore the gene expression to
normal cellular levels or overexpress the gene in a
population of malignant cells. In a preferred
embodiment, the gene to be expressed is MAFbx.

In another embodiment, nucleic acid molecules may be
20   used to decrease expression of genes found to be down-
regulated after successful imatinib treatment (preferably
SPRY4A), as yet another means to treat a malignancy (see
Example 1, Tables 2-4). In this embodiment,
oligonucleotides are targeted to specific regions of the
25   genes that are critical for gene expression. The use of
antisense oligonucleotides to decrease expression levels
of a pre-determined gene is known in the art. In a
particular embodiment, such antisense oligonucleotides
are modified in various ways to increase their stability
30   and membrane permeability, so as to maximize their
effective delivery to target cells in vitro and in vivo.
Such modifications include the preparation of

phosphorothioate or methylphosphonate derivatives, among
many others, according to procedures known in the art.

In another embodiment, siRNAs directed to the genes
may be employed to down regulate the expression of the
5   genes. Selection of a suitable small interfering RNA
(siRNA) molecule requires knowledge of the nucleotide
sequence of the target mRNA, or gene from which the mRNA
is transcribed. The siRNA molecules of the invention are
typically between 12-30 nucleotides in length. More
10   preferably, siRNA molecules are about 20, 21, 22, and 23
nucleotides in length. The siRNA molecules may comprise
a sequence identical or at least 90% identical to any
portion of the target gene whose expression is to be
modulated including coding and non-coding sequences. A
15   common method for identifying an siRNA target site within
the target gene comprises scanning for AA dinucleotide
sequences downstream of the AUG start codon and
identifying the AA dinucleotide and the adjacent 3' 19
nucleotides as an siRNA target (see, for example, Ambion®
20   Guidelines; Austin, TX; Ausubel et al., eds. Current
Protocols in Molecular Biology, John Wiley and Sons,
Inc., (1995)). Optionally, the identified sequence may
be searched against a genome database such as GenBank®
(maintained by The National Center for Biotechnology
25   Information; NCBI) by a program such as BLAST® (Basic
Local Alignment Search Tool) to ensure the sequence does
not have significant homology to other genes.

30   The following examples present further detail
regarding the practice of the instant invention. These
examples are provided for illustrative purposes only and

are not intended to limit the scope of the invention in any way.

EXAMPLE 1

5 **Expression profiling of GIST cells treated with Imatinib using cDNA microarrays**

Materials and Methods:

GIST cell culture

GIST882 cells were maintained in F-10 media
10 supplemented with 15% FCS, 1% Bovine Pituitary Extract (Invitrogen, Carsbad, CA), 0.5% MITO+ Serum Extender (Becton Dickinson, Bedford, MA) and L-glutamine.

GIST cells proliferation and apoptosis assessment

15   For growth analysis, GIST882 cells were seeded at
6.5×10^5 cells/60-mm dish. Imatinib (provided by Novartis Oncology and dissolved in water) was added directly to the media to achieve a final concentration of 1 or 10 µM. Cells were refed with conditioned media
20 containing imatinib or with conditioned media without drug every 12 h. Cells were harvested and stained for the cell number, cell viability, and induction of apoptosis using Guava ViaCount and Guava Nexin reagents (Guava Technology Inc., Burlingame, CA). The cells were
25 counted using a Guava Personal Cytometer, and the data were analyzed using the Guava CytoSoft software package.

Cell Cycle Analysis

Cells were trypsinized, centrifuged, and fixed in
30 70% ethanol at 4°C. Cell pellets were resuspended in 50 µg/ml propidium iodide in PBS for 30 min at 4°C. The stained cells were analyzed by flow cytometry performed

on a FACScan, and the data were analyzed with Cell Quest
software (Becton Dickinson).

GIST cells treatment with Imatinib

5   Imitanib mesylate was dissolved in water at a stock
concentration of 10 mM. GIST882 cells were cultured to
60-70% confluency. Forty-eight hours prior to the
treatment, cells were refed with complete media.
Imitanib was added directly to the media to achieve the
10   final concentration of either 1 μM or 10 μM.

Total RNA preparation

      Imatinib treated and control GIST882 cells were
harvested simultaneously at 60-80% confluency. Total RNA
15   was isolated using guanidinium/isothiocyanate/
phenol/chloroform method as previously described [Schultz
et al., Cancer Res 56, 1997-2002. (1996)]. Total RNA was
separated by agarose gel electrophoresis and visualized
by ethidium bromide staining to check for integrity. 100
20   μg of total RNA was DNase treated using "DNA free" kit
(Ambion, Austin, TX) according to manufacturer's
specifications. Following DNase treatment the RNA was
quantified and evaluated for integrity by agarose
electrophoresis.

25

Microarray preparation

      10,367 human cDNA fragments corresponding to known
genes and ESTs from the I.M.A.G.E. consortium library
(Research Genetics) were amplified by polymerase chain
30   reaction (PCR), purified by isopropanol precipitation and
resuspended in 50% DMSO at a concentration of 150 ng/μl.
Arrays were spotted on GeneMachine Omnigrid arrayer
(GeneMachine, San Carlos, CA) using poly-Lysine coated

glass slides. Slides were baked for 3 hours at 80°C in a
vacuum oven, crosslinked in UV light (90 mJ) in
Stratalinker (Stratagene, La Jolla, CA) and processed as
described in Shalon et al., Genome Research 6 (7), 639-
5   645, (1996) and DeRisi et al., Science 278 (5338), 680-
686, (1997).

Preparation and hybridization of the probe cDNA
        Fifteen micrograms of total RNA were reverse
10   transcribed and amino allyl dUTP was incorporated in a
reaction containing 500 ng oligo (dT) primers, 1x first
strand buffer (Invitrogen, Carlsbad, CA), 0.01 M DTT, 500
μM each of dATP, dCTP, dGTP, and dTTP/aadUTP (2:3 ratio)
40 Units of rRNasin (Promega, Madison, WI) and 200 Units
15   of SuperScript II reverse transcriptase (Invitrogen,
Carlsbad, CA). After brief denaturation and annealing of
the primers at 70°C for 8 min, the reaction was incubated
at 42°C for 2 hours, followed by alkali hydrolysis of RNA
and cDNA purification using Microcon-30 columns
20   (Millipore, Bedford, MA) according to the manufacturer's
instructions. The cDNA was then labeled with either Cy3
or Cy5 dyes by a coupling reaction using FluoroLinkTM Cy3
and Cy5 monofunctional dyes (Amersham Pharmacia Biotech,
Piscataway, NJ) according to manufacture's
25   specifications. Probes were purified using StrataPrep
PCR Purification Kit (Stratagene, La Jolla, CA). Two of
the samples (one labeled with Cy3 and one with Cy5) were
combined, denatured, pre-annealed in the presence of 10
μg of Cot-1 DNA (Invitrogen, Carlsbad, CA) and 10 μg of
30   poly-dA DNA, and hybridized to the cDNA microarrays
overnight at 42°C in hybridization buffer (25% formamide,
5x SSC, 0.1% SDS and 100 μg/ml sonicated salmon sperm
DNA). After hybridization, the arrays were washed 2

times in washing buffer A (2 x SSC, 0.1% SDS) at 42°C for
5 minutes and then 3 times in washing buffer B (0.1 x
SSC, 0.1% SDS) at room temperature for 10 minutes. The
slides were briefly dipped into distilled water and dried
5   in a stream of nitrogen before scanning.

Intensity extraction and data analysis

   Images were obtained by scanning the arrays in
Affymetrix 428 scanners. Signal intensities for Cy3- and
10 Cy5-labeled probes were extracted by ImaGene software
package version 4.2 (BioDiscovery, Inc., Marina Del Rey,
CA) using default settings and auto segmentation. Mean
intensities for signal and background as well as quality
characteristics ("empty" or "poor") of the spots were
15 obtained at this time. The threshold for empty spots was
achieved by raising threshold to a point when all blank
spots were flagged. The formula for determining this
value is as follows: if $(M_S-M_B)/\sigma_B <$ threshold, then the
spot is flagged, where M_S is the mean of signal, M_B is the
20 mean of background and σ_B is the Standard Deviation of the
background. The "poor" spots were calculated using the
following formula: if $\sigma_S/M_S >$ threshold, then the spot is
flagged (σ_S is the Standard Deviation of the signal and MS
is the Mean of signal). The threshold was set at 0.4 to
25 determine "poor" spots. The data were then analyzed
using GeneSight software package version 3.0.4
(BioDiscovery, Inc., Marina Del Rey, CA). Data
preparation consisted of the following steps: 1)
background correction was performed by subtracting the
30 local group median (clique size = 5) background from the
signal of each spot; 2) spots that were flagged as
"empty" and "poor" were omitted from the analysis; 3) the
data were normalized using Piece-wise linear

normalization, which was done using 10 bins and a minimum of 100 elements in each bin; 4) the intensity ratio of two channels was calculated; 5) the $\log_2$ was calculated for each ratio; and 6) the two channels were normalized

5 by subtracting the $\log_2$ of the mean of the intensity of the total signal from each individual spot intensity. At least two "flip-dye" experiments were performed for each time point and the mean of the repeated experiments were used in the final data analysis. Finally, the mean ratio

10 for each spot was calculated for all time points and the standard deviation and coefficient of variance were calculated for these values.

cDNA microarray clone verification and sequence analysis

15 cDNA fragments of interest were obtained from original stock plates used for microarray fabrication by either plasmid isolation or direct amplification of the fragments from the bacteria. Clones were re-sequenced and the correct annotation and homology was identified

20 using the basic local alignment search tool (BLAST, NCBI) against GenBank/EMBL database. DNA and amino acid sequence analyses were performed with the Wisconsin Package version 9.1 (Genetics Computer Group).

25 *Northern Blotting*

Fifteen micrograms of total RNA was used for standard Northern blot analysis, as previously described [Schultz et al., Cancer Res 56, 1997-2002. (1996)]. Blots were hybridized with a 1,454 bp ^{32}P-labeled SPRY4A

30 cDNA probe, corresponding to nucleotides 2594-4048 of GenBank entry AF227516.

Reverse transcription of RNA

      Two micrograms of DNase treated RNA were reverse transcribed using M-MuLV reverse transcriptase (NEB, Beverly, MA) in a 20 μl reaction containing 1 x RT Buffer

5   (NEB, Beverly, MA), 0.5 mM each dNTPs, 4 μM oligo-dT (16-18) primer, 10 units RNase inhibitor (Promega, Madison, WI) and 200 units of reverse transcriptase. Primers were pre-annealed for 10 min at 70°C, and the reaction incubated for 1 hr at 42°C followed by enzyme

10   inactivation for 10 min at 90°C.

Polymerase chain reaction

      DNA fragments of SPRY4A/C, SPRY4A and β-actin were amplified by polymerase chain reaction (PCR) using cDNAs

15   obtained by reverse transcription of mRNA from GIST cells and tumor biopsies as a template and the following sets of primers: SPRY4A – forward 5'-CCGTTCTGTGGAGAGTCGATTTAC-3' (SEQ ID NO: 1), reverse 5'-GTCCCTCAGTGGCTCTCGACT-3' (SEQ ID NO: 2); Sprouty4A/4C (isoform determining) –

20   forward 5'-ACCATCCTACCCATTGACCA-3' (SEQ ID NO: 3), reverse 5'-GGCTTCGACACAAACTGTCA-3' (SEQ ID NO: 4); β-actin – forward 5'-CTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 5), reverse 5'-CATGATGGAGTTGAAGGTAGTTTCGT-3' (SEQ ID NO: 6). PCR was performed in a reaction volume of 30 μl

25   containing cDNA from 1 μl of the reverse transcription reaction described above as a template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin, 0.5 μM of both the forward and reverse primer, 60 μM of each deoxyribonucleotide, 5% dimethyl sulfoxide, and 0.5U

30   Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Following an initial denaturation step at 95°C for 5 min, DNA was amplified through 21, 23, 25, and 27 cycles consisting of 5 sec denaturing at 95°C, 60 sec annealing

at 55°C, and 90 sec extension at 72°C. The products were
resolved on a 1.5% agarose gel and were visualized by UV
light following staining with Ethidium Bromide. Images of
the gels were obtained using Alpha Imager 2200

5   documentation and analysis system (Alpha Innotech, San
Leandro, CA). Images were captured within linear dynamic
range and controlled for white color saturation. DNA
bands were quantified using Alpha Imager v5.5 software
package and Fuji Image Gauge v3.11 software package (Fuji

10 Photo Film Co., Ltd).

Results:

    The response of GIST882 cells to imatinib was
assessed *in vitro*. Both 1 and 10 μM imatinib

15 dramatically inhibited cell growth within 24-48 h
after exposure (Fig. 2A). However, GIST cells treated
with imatinib show no significant increase (<3% of total
cell number) in either early or late apoptosis during 96-
h treatment period as determined by annexin-V and 7

20 amino-actinomycin D staining. The fraction of viable
cells was 93 ± 5% for both treated and untreated cells
during entire course of treatment (data not shown). A
cell cycle analysis of drug-treated cells was also
performed and a 57% (15.5% versus 6.6% of total cell

25 amount) decrease in the amount of cells in S phase with
addition of 1 or 10 μM of imatinib was found (Table 1).
The variation of the total number of cells in S phase was
<5% for independently repeated experiments.

30

Tabl 1

	72 hours			96 hours		
	G0-G1	G2-M	S	Go-G1	G2-M	S
Untreated	74.72%	9.28%	15.99%	74.33%	10.63%	15.04%
1μM imatinib	84.21%	8.53%	7.25%	79.29%	13.48%	7.22%
10μM imatinib	85.70%	7.87%	6.43%	80.87%	12.23%	6.90%

Table 1: Percentage of GIST882 cells in indicated phase of cell cycle at the 72 and 96 hour time points.

To identify potential imatinib-specific genetic targets, a human GIST cell line (GIST882) was treated with 10 μM imatinib for 0, 6, 24, or 48 hr. RNA samples labeled with Cy3- or Cy5-dyes were hybridized to 10,368-element cDNA microarrays containing known genes and ESTs. 148 genes or ESTs (expressed sequence tags) were found to be differentially regulated in at least one timepoint with 95% confidence (Table 2). 62 genes displayed significant down-regulation at all time points tested (Table 3). 14 genes were identified, which displayed a significant change (at least 2.5-fold) in expression levels at all time points following treatment (Table 4). Two genes were upregulated and 12 genes were down-regulated (see Table 4). The mean intensity ratio was calculated for all spots during the course of drug treatment and it was found that out of the 12 genes which showed decreased expression following drug treatment, only one transcript was dramatically down-regulated at each time point (from 4- to 13-fold) (Fig. 1). Therefore, the studies were focused on this gene.

Table 2

Upregulated Genes

AA009593--ESTs
5   AA011673--ESTs, Weakly similar to ALU8_HUMAN ALU SUBFAMILY SX SEQUENCE
          CONTAMINATION WARNING ENTRY [H.sapiens]
      AA057425--myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog);
          translocated to, 2
      AA100674 | AI732414--ESTs
10   AA148532--Homo sapiens, Similar to CG7083 gene product, clone MGC:10534, mRNA, complete cds
      AA416785--heterogeneous nuclear ribonucleoprotein A1
      AA423978--ESTs, Weakly similar to JM27 [H.sapiens]
      AA427528--zinc-finger protein ZBRK1
      AA454597--golgi membrane protein GP73
15   AA455497--protein tyrosine phosphatase, receptor type, C
      AA457700--stearoyl-CoA desaturase (delta-9-desaturase)
      AA463256--ESTs
      H09124--Homo sapiens cDNA: FLJ23573 fis, clone LNG12520
      H09164--Homo sapiens cDNA: FLJ22030 fis, clone HEP08669
20   H25042--chromosome 8 open reading frame 1
      N21407--activated RNA polymerase II transcription cofactor 4
      N23606--SRY (sex determining region Y)-box 4
      N30157--ESTs
      N50935--hypothetical protein ASH1
25   N69100--EST
      R97944--zinc finger protein 26 (KOX 20)
      T41066--ESTs
      W69669--chromosome 21 open reading frame 4

30   Downregulated Genes

      AA004595--ESTs
      AA011041--ESTs
      AA022558--Homo sapiens cDNA: FLJ22120 fis, clone HEP18874
35   AA022949--fibroblast growth factor 18
      AA043878--KIAA1151 protein
      AA055491--transmembrane 4 superfamily member (tetraspan NET-2)
      AA055971--KIAA0810 protein
      AA083207--EST
40   AA101833--ESTs
      AA128617--hypothetical protein FLJ20898
      AA131530--ESTs
      AA133215--receptor (calcitonin) activity modifying protein 1
      AA133778--ESTs
45   AA149637--transducin (beta)-like 1
      AA158375--SRp25 nuclear protein
      AA164229 | AI732625--ESTs
      AA181898--hypothetical protein FLJ20186
      AA206050--ESTs
50   AA283599--CGI-94 protein
      AA286905--checkpoint with forkhead and ring finger domains
      AA287828--up-regulated by BCG-CWS
      AA398262--ESTs, Weakly similar to AAB47496 NG5 [H.sapiens]
      AA401470--hypothetical protein RP4-622L5
55   AA412477--EST
      AA417011--EST
      AA418418--hypothetical protein FLJ10055
      AA425382--sprouty (Drosophila) homolog 4

AA427395--Homo sapiens dpy-30-like protein mRNA, complete cds
AA427767 | AI732744--RNA binding motif protein 14
AA427778--complement C1r-like proteinase precursor,
AA432064--ESTs
5   AA446893--Homo sapiens cDNA FLJ10705 fis, clone NT2RP3000850
AA446906--solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kD),
          member 17
AA447746--hypothetical protein
AA453287--hypothetical protein MGC5306
10   AA453616--Homo sapiens AKAP-associated sperm protein (ASP) mRNA, complete cds
AA453994--NPD007 protein
AA454149--EST
AA455042--chemokine-like factor, alternatively spliced
AA455275--serologically defined breast cancer antigen NY-BR-87
15   AA455291--ESTs
AA457138--ESTs
AA463444--RB-associated KRAB repressor
AA463516--lysophosphatidic acid acyltransferase-delta
AA464578--Homo sapiens, clone MGC:3182, mRNA, complete cds
20   AA464736--Homo sapiens, clone MGC:10710, mRNA, complete cds
AA478630--Huntingtin interacting protein C
AA479155--ESTs
AA485357--ESTs
AA486445--hypothetical protein FLJ10120
25   AA487465--
AA487527--KIAA0937 protein
AA488650--TRAF and TNF receptor-associated protein
AA598625--solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
AA599043--ESTs
30   AA609422--EST
AA620488--
AA625765--hypothetical protein MGC2594
AA757671--
AA778683--ESTs
35   AA876219--phosphodiesterase 2A, cGMP-stimulated
AA932870--zinc finger protein 143 (clone pHZ-1)
AI015536--mitochondrial ribosomal protein L12
AI075273--phosphodiesterase 7A
AI094859--engrailed homolog 2
40   AI129421--
AI218900--H4 histone family, member I
AI337297--tumor suppressing subtransferable candidate 3
H01164--serine/threonine kinase 17a (apoptosis-inducing)
H09082--ESTs
45   H09241--ESTs
H11051--copine VI (neuronal)
H11918--KIAA0534 protein
H16789--CGI-152 protein
H18934--Human DNA sequence from clone 321D2 on chromosome 16. Contains a gene for a
50   Ribosomal Large Subunit Pseudouridine Synthase (EC 4.2.1.70, Pseudouridylate Synthase,
          Uracil Hydrolase) LIKE protein, a gene for a novel protein similar to replication facto
H28794--ESTs
H49519--ESTs
H52062--hypothetical protein FLJ22313
55   H63534--methylmalonate-semialdehyde dehydrogenase
H84926--
H92504--hypothetical protein
N21081--HMBA-inducible
N22836--ESTs

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        N29696--EST
        N34470--ESTs
        N38992--GATA-binding protein 4
        N39077--ESTs
   5    N39573--KIAA1183 protein
        N48070--ESTs
        N51651--KIAA0909 protein
        N56906--EST
        N62376--ESTs
  10    N63375--EST
        N68864--ESTs, Weakly similar to ALU8_HUMAN ALU SUBFAMILY SX SEQUENCE
                CONTAMINATION WARNING ENTRY [H.sapiens]"
        N70837--KIAA1275 protein
        N73571--
  15    N81032 | AI822124--
        N93057 | AI668684--ESTs
        R16053--HMG-box containing protein 1
        R19306--nuclear factor I/X (CCAAT-binding transcription factor)
        R34224--ESTs
  20    R43308--Homo sapiens mRNA; cDNA DKFZp434D0720 (from clone DKFZp434D0720)
        R44562--KIAA0564 protein
        R78530 | R78490--Homo sapiens cDNA: FLJ22380 fis, clone HRC07453, highly similar to
                HUMCLPB Homo sapiens CLP mRNA
        R85261--ESTs
  25    R86847--FH1/FH2 domain-containing protein
        T49802--hypothetical protein MGC2477
        T55569--hypothetical protein FLJ11773
        T60082--CpG binding protein
        T82453--ESTs, Weakly similar to T12482 hypothetical protein DKFZp564P0662.1 [H.sapiens]
  30    T83646--phosphoglycerate dehydrogenase
        T94848 | AI732248--ESTs
        T96985--hypothetical protein MGC4692
        W19822--semaphorin Y
        W37689--ESTs, Weakly similar to weak similarity to TPR domains [C.elegans]
  35    W48838--hypothetical protein MGC4175
        W56308--EST
        W67228--ESTs
        W84774--ESTs
        W87801--ESTs
  40    W87939--ESTs
        W89128--ESTs
        W93024--Homo sapiens clone IMAGE:112574 mRNA sequence
        W93943--ESTs
        W94591--ESTs
  45    _____
```

Table 2: GenBank Accession numbers and brief
descriptions, where available

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  50
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Table 3

AA039851--protein tyrosine phosphatase type IVA, member 3
AA417373--2,4-dienoyl CoA reductase 2, peroxisomal
AA446906--solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kD),
5   member 17
AA464578--Homo sapiens, clone MGC:3182, mRNA, complete cds
AA598625--solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
AA776942--coatomer protein complex, subunit epsilon
AA876219--phosphodiesterase 2A, cGMP-stimulated
10 AI190798--keratin, hair, acidic,2
AI218900--H4 histone family, member I
H98755--ESTs, Weakly similar to unknown protein [R.norvegicus]
N62400--heat shock 90kD protein 1, alpha
N62979--ESTs, Highly similar to T00391 hypothetical protein KIAA0612 [H.sapiens]
15 N71692--ESTs, Weakly similar to ALUC_HUMAN [H.sapiens]"
R78530 | R78490--Homo sapiens cDNA: FLJ22380 fis, clone HRC07453, highly similar to
       HUMCLPB Homo sapiens CLP mRNA
T82453--ESTs, Weakly similar to T12482 hypothetical protein DKFZp564P0662.1 [H.sapiens]
W57983--pinin, desmosome associated protein
20 AA004595--ESTs
AA043878--KIAA1151 protein
AA128617--hypothetical protein FLJ20898
AA133215--receptor (calcitonin) activity modifying protein 1
AA158375--SRp25 nuclear protein
25 AA164229 | AI732625--ESTs
AA255876--zinc finger protein ANC_2H01
AA293215--ubiquinol-cytochrome c reductase core protein I
AA398332--EST
AA401341--chromosome 16 open reading frame 5
30 AA412477--EST
AA417011--EST
AA425382--sprouty (Drosophila) homolog 4
AA427570--NADH dehydrogenase (ubiquinone) flavoprotein 1 (51kD)
AA447746--hypothetical protein
35 AA453994--NPD007 protein
AA455523--
AA457138--ESTs
AA460274--DKFZP434P106 protein
AA485357--ESTs
40 AA487301--ESTs
AA487465--
AA490249--arginine-glutamic acid dipeptide (RE) repeats
AA598781--matrix Gla protein
AA609422--EST
45 AI015536--mitochondrial ribosomal protein L12
AI125840--leucine-rich neuronal protein
H48165--ESTs
H49519--ESTs
H63534--methylmalonate-semialdehyde dehydrogenase
50 N21385--choroideremia-like (Rab escort protein 2)
N34470--ESTs
N51651--KIAA0909 protein
N62908--ESTs
N63375--EST
55 N67305--ESTs
N70837--KIAA1275 protein
N81032 | AI822124--
R19306--nuclear factor I/X (CCAAT-binding transcription factor)

R44840--ESTs
T55569--hypothetical protein FLJ11773
T64323--
T83646--phosphoglycerate dehydrogenase
5   W32281--CGI-39 protein; cell death-regulatory protein GRIM19
W74725--Homo sapiens mRNA; cDNA DKFZp434A1319 (from clone
         DKFZp434A1319); complete cds
W87939--ESTs

10   Table 3: GenBank Accession numbers and brief
     descriptions, where available

Table 4

ACC	Clone ID	6 hours	12 hours	24 hours	48 hours	Mean ratio	CV
AF227516	Sprouty (Drosophila) homolog 4	-3.31	-1.91	-3.73	-2.38	-2.83 ± 0.83	0.29
NM_031866	Frizzled (Drosophila) homolog 8	-2.22	-1.39	-2.54	-0.97	-1.78 ± 0.72	0.41
AF335324	RTP801	-2.11	-1.17	-1.67	-1.35	-1.58 ± 0.41	0.26
AC106791	EST chromosome 5	-1.89	-0.67	-2.29	-1.46	-1.58 ± 0.69	0.44
NM_024600	Hypothetical protein FLJ20898	-1.63	-0.45	-2.5	-1.49	-1.52 ± 0.84	0.55
AC124312	EST (chromosome15)	-0.93	-1.43	-1.72	-1.7	-1.45 ± 0.37	0.26
BC020567	GEF (Rho/Rac guanine exchange factor)	-1.95	-0.8	-1.39	-1.49	-1.41 ± 0.48	0.34
T82453	EST	-0.86	-1.03	-2.42	-0.99	-1.32 ± 0.73	0.55
AA487465	EST	-0.87	-0.81	-1.53	-1.99	-1.30 ± 0.57	0.44
AA598625	Solute carrier family 1	-0.34	-0.88	-2.93	-1.01	-1.29 ± 1.13	0.88
NM_002599	Phosphodiesterase 2A, cGMP-stimulated	-0.45	-0.72	-2.63	-1.25	-1.26 ± 0.97	0.77
N49836	EST	-1.57	-0.96	-0.96	-1.51	-1.25 ± 0.34	0.27
AY059629	MAFbx	1.81	1.13	1.61	0.72	1.32 ± 0.49	0.37
H70163	EST	0.92	2.16	1.93	0.49	1.38 ± 0.80	0.58

Table 4: cDNA microarray analysis of imatinib treated
15   GIST 882 cells. Shown are the log_2 intensity ratios for
     each time point and the overall mean log_2 ratio for each
     gene. StDev = Standard deviation, CV = Coefficient of
     variance, EST = Expressed sequence tag, ACC = GenBank
     Accession Number.
20

     Upon sequencing and database evaluation, it was

     found that the sequence of the spotted cDNA fragment

     matched two isoforms of Sprouty4, 4A and 4C (Figs. 2B).

     SPRY4A (GenBank Accession No. AF227516) and SPRY4C

25   (GenBank Accession No. AF227517) represent alternatively·

     spliced forms of the same gene. The SPRY4A transcript

     encodes for a 322 amino acid protein, while SPRY4C

transcript encodes for a 106 amino acid polypeptide (Fig. 2B). The SPRY4A transcript includes all of the SPRY4C sequence with extended amino and carboxy termini.

In support of the microarray results, a cDNA probe

5   was derived that would detect both the ~5,000 base (SPRY4A) and the ~7,000 base (SPRY4C) transcripts of Sprouty4. Northern blot analysis indicated that SPRY4A was down-regulated following treatment with 10 μM imatinib (Fig. 2C). In comparison, SPRY4C transcript was

10   not detected by Northern blotting (data not shown). A set of oligonucleotide PCR primers were designed that can differentiate between the two Sprouty4 transcripts (Fig. 2B). The oligonucleotide primer pair 1F/1R is predicted to yield a RT-PCR product of ~500 bp that would represent

15   both SPRY4A and 4C. In comparison, primer pair 2F/2R would yield a fragment of 812 bp for SPRY4A and a 358 bp fragment for SPRY4C (Fig. 2B). The RT-PCR analysis confirmed that SPRY4A, and not 4C was constitutively expressed in GIST cells and exhibited dramatic down-

20   regulation after drug treatment (Fig. 2D).

Example 2
Imatinib inhibits SPRY4A expression through down-regulation of c-KIT, ERK1/2 and AKT

25   Materials and Methods:
Cell lysate preparation, SDS-PAGE and Western blot analysis

Anti-β-actin was purchased from Sigma (St. Louis, MO) and was probed in 1:5000 dilution in 5% dried milk.

30   Anti-phospho-c-Kit (Tyr719) was purchased from Cell Signaling (Beverly, MA) and was probed in 1:500 dilution in 5% BSA. Anti-c-KIT was purchased from Santa Cruz (Santa Cruz, CA) and was used in 1:100 dilution in 5%

dried milk. Anti-phospho-ERK1/2 was purchased from Cell
Signaling (Beverly, MA), anti-ERK1, and anti-ERK2 were
obtained from Santa Cruz Biotechnology Inc (Santa Cruz,
CA), anti-AKT and anti-AKT/phosphoThr308 antibodies were
5   obtained from Cell Signaling Technology (Beverly, MA) and
were probed in 1:1000 dilution in 5% BSA.

Cells at 60-70% confluence were washed twice with
ice-cold D-PBS before scraping on ice with Lysis Buffer
[20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM Na-
10   pyrophosphate; 1 mM Na-β-glycerophosphate; 5 mM NaF, 1 mM
Na_3VO_4, 1 mM PMSF, 1% Triton X-100, and 1 tablet of
protease inhibitor cocktail (Roche, Indianapolis, IN) per
40 ml lysis buffer]. Cellular debris was removed by
centrifugation (14,000 x g for 15 min at 4°C), and
15   quantitation of protein was performed using a
bicinchoninic acid/copper (II) sulfate assay (Sigma, St.
Louis, MO). Fifty micrograms of total protein from cell
extracts and tissues were separated by standard SDS-PAGE
and transferred to Immobilon-P (polyvinylidene
20   difluoride; Millipore, Bedford, MA). Prior to antibody
probing, the membranes were blocked in 5% dried milk.
The primary antibodies were diluted 1:1000 (unless other
specified), and the HRP-conjugated secondary antibodies
were diluted 1:10,000 (Amersham, Piscataway, NJ). NEN
25   Renaissance Enhanced Luminol Reagents (Boston, MA) were
used as substrates for detection. For reuse of the same
membrane with another primary antibody, the membrane with
stripped with Restore Western Blot Stripping buffer
(Pierce, Rocklord, IL).

30

Results:

GIST882 cells were examined for activation of c-KIT,
ERK1/2 and AKT using anti-phosphorylated antibodies. c-

KIT, ERK1/2 and AKT were found to be constitutively

activated in exponentially growing GIST882 cells (Fig.

3A). The inhibitory efficacy of the drug on the activity

of c-KIT, ERK1/2 and AKT was then evaluated with

5   different drug concentrations (1 and 10 μM of imatinib)

at various time intervals. It was found that either drug

concentration resulted in the loss of phosphorylated c-

KIT within 30 minutes without affecting the total level

of KIT protein (Fig. 3A). Imatinib also potently

10   inhibited the constitutive activation of ERK1/2

(ppERK1/2) without affecting total cellular levels of

ERK1/2 (Fig. 3A). Inhibition of AKT took place within 30

minutes for 10 μM of imatinib and 1 hour for 1 μM without

affecting the total level of AKT (Fig. 3A). Imatinib

15   effects on SPRY4A mRNA expression in GIST882 were

evaluated next. At both concentrations of drug, SPRY4A

levels were noticeably decreased by 3 hrs and virtually

undetectable by 6 hrs (Fig. 3B). Therefore, it was shown

in GIST882 cells that imatinib treatment resulted in

20   decreased autophosphorylation of the mutant c-KIT

polypeptide by inhibiting c-KIT kinase activity rather

than by down-regulating expression of the c-KIT protein.

Furthermore, this inhibition leads to down-regulation of

the activation of ERK1/2 and AKT and decreased levels of

25   SPRY4A mRNA.

Example 3
SPRY4A expression is regulated by the ERK1/2 signaling pathway independent of AKT

30   Materials and Methods:

GIST cells treatments with MEK inhibitor

The MEK1/2 inhibitor U0126 was purchased from

Promega (Madison, WI) and dissolved in DMSO at a stock

concentration of 15 mM. GIST cells were cultured to 60-
70% confluency. Forty-eight hours prior to the
treatment, the media was replaced. MEK inhibitor was
added directly to the media to achieve the final
5 concentration of 1 μM, 10 μM and 30 μM.

Results:

   Previous studies have shown that the ERK pathway
positively regulates the expression of the Sprouty genes
10 in mouse cells, and that in a limited number of tumor
cell lines which exhibit constitutive activation of ERKs,
SPRY1 and/or SPRY2 mRNA is elevated [Ozaki et al.,
Biochem Biophys Res Commun 285, 1084-8. (2001)]. To
determine if ERK1/2 pathways also regulated SPRY4A in
15 tumor cells, the GIST882 line was treated with varying
concentrations of U0126, a MEK inhibitor, for 6 hrs and
24 hrs and the expression of SPRY4A was evaluated. It
was found that SPRY4A levels were decreased ~90% and ~70%
by 6 and 24 hrs, respectively, at a concentration of 30
20 μM U0126 (data not shown). Lower doses of U0126 had
minimal effect on SPRY4A levels at either of the time
points (data not shown). The levels of constitutively
activated ERK1/2 following treatment with 30 μM U0126 for
30 min, 1, 3, and 6 hrs were evaluated next. This
25 treatment resulted in a complete suppression of activated
ERKs by 30 min; expression of SPRY4A was reduced by 3 hrs
and was nearly absent by 6 hrs (Fig. 4A and 4B). The
level of SPRY4A down-regulation was comparable to that
seen with imatinib, except that the duration of
30 suppression with a single dose of imatinib was more
prolonged (data not shown). Interestingly, U0126
inhibits phosphorylation of AKT shortly after treatment
but this effect was transient and the amount of phospho-

AKT returned to the initial level within 3 hours of
treatment (Fig. 4A). Furthermore, the presence of U0126
did not affect the levels of total AKT. These results
further confirm that constitutive activation of ERK1/2
5   through the c-KIT pathway leads to expression of SPRY4A
and that this process may be independent of AKT
signaling.

Example 4

10   **SPRY4A and MAFbx expression in clinical GIST samples**
Materials and Methods:
Clinical samples

Tumor specimens were obtained from seven patients
enrolled in the CSTI571-B2222 clinical trial sponsored by
15   Novartis Oncology. Eligibility criteria included
histological confirmation of GIST with documentation of
c-KIT expression, as well as evidence of unresectable
recurrent and/or metastatic disease. After signing
informed consent, patients were evaluated to confirm
20   acceptable hematologic, renal and hepatic function.
Patients were then randomized to receive 400 mg or 600 mg
per oral once daily dosing of imatinib. Ultrasound-
guided 14 gauge core biopsies of non-necrotic tumor were
obtained prior to therapy initiation and while on drug
25   for between 1-28 days. Specimens were flash frozen and
kept in liquid N_2. Patients were monitored for response
to imatinib mesylate at 4-12 week intervals.

Patients #1-3 had GIST of either the stomach or the
small bowel and had a partial response to imatinib
30   treatment defined as greater than or equal to 50%
decrease from baseline in the sum of products of
perpendicular diameters of all measurable lesions.
Patient #4 had a partial response to imatinib initially

but developed disease progression 3 months after
initiation of the treatment. The patient was taken off
drug and a core tumor biopsy was obtained. Patients #5
and #6 presented with large intestine and gastric GIST,

5   respectively, and they had no response to imatinib
treatment. Patient #5 remained with stable disease and
Patient #6 developed disease progression. Additional
tumor was obtained from patient #6 during palliative
surgical resection approximately one week after

10   discontinuing drug therapy. Patient #7 had two
measurable abdominal tumors, one of which responded to
therapy (R) where as the other progressed (NR).

        Total RNA from the tumor specimens was isolated
using Trizol reagent (Invitrogen, Carlbad, CA) using

15   manufacturer's protocol. See other examples for RT-PCR
conditions. Protein extracts from a patient with a
myxoid extremity liposarcoma and GIST patient #6 tumor
were prepared as described above.

20   Results:
        Core biopsy tumor specimens taken from imatinib-
treated GIST patients (as described in Methods) were
evaluated for expression of SPRY4A by RT-PCR analysis.
The expression levels of SPRY4 were dramatically

25   decreased following imatinib therapy in tumors from
patients (#1, 2, 3, and 7(R)) who showed a favorable
clinical response to the drug (Fig. 5A). In comparison,
the imatinib-resistant patients (#5, 6, and 7 (NR))
expressed similarly high levels of SPRY4A both pre- and

30   post-treatment. In the patient (#4) who initially
responded to the drug treatment but subsequently
relapsed, the SPRY4A levels in the tumor decreased
dramatically for the duration of the favorable clinical

response but returned to the pre-treatment levels during
disease progression (Fig. 5A).

The expression level of MAFbx in clinical samples
was also assessed (patients 1, 4, 6, and 7). In patients
5 1 and 7(R), both of whom showed favorable clinical
responses, high levels of MAFbx transcripts were detected
in the treated tumor. Notably, the NR tumor from patient
7 showed low/minimal levels of MAFbx. In the tumor
sample from patient 4, the level of MAFbx increased
10 during the response but returned to pretreatment level
during the disease progression. In patient 6, who never
responded to the drug, the level of MAFbx was not changed
(i.e., not detected) in the paired samples (Fig. 5B).

In patient #6, sufficient tissue from the non-
15 responding tumor was available to evaluate the c-KIT, AKT
and ERK1/2 protein levels. A western blot of the drug
resistant GIST indicated a continued expression of
activated forms of c-KIT, AKT and ERK1/2 during imatinib
therapy (Fig. 6A, B, and C). In comparison, a control
20 specimen taken from an untreated patient with the
histological diagnosis of myxoid liposarcoma, a sarcoma
histotype that is clinically unresponsive to imatinib,
failed to express detectable levels of c-KIT (Fig. 6A).
Importantly, in this control liposarcoma tissue, ERK1/2
25 was constitutively activated (Fig. 6B) and SPRY4A was
expressed at a level comparable to a typical GIST
specimen (data not shown). These results suggest that
constitutive activation of ERK1/2 in GISTs and in a
liposarcoma, whether c-KIT-dependent or -independent,
30 contribute to expression of SPRY4A. It was determined
that the tumor from patient #6 possessed an in frame
mutation (1530ins6) in exon 9 of c-KIT. Together, these
results suggest that SPRY4A is regulated in GISTs by the

c-KIT and ERK1/2 pathways and can be used as a genetic
marker of clinical response to imatinib treatment for
these tumors. This exon 9 insertion of c-KIT may be
associated with GIST cells to respond which are
5 refractory to treatment.

Example 5

Imatinib responses of other genes

<u>Materials and Methods</u>:

10 *Reverse Transcription of RNA*

        Two µg of DNase treated RNA were reverse transcribed
using Moloney murine leukemia virus reverse transcriptase
(New England Biolabs, Beverly, MA) in a 20-µl reaction
containing 1X reverse transcription buffer (NEB), 0.5 mM
15 each dNTP, 4 µM oligo(dT)$_{16-18}$ primer, 10 units of RNase
inhibitor (Promega), and 200 units of reverse
transcriptase. Primers were preannealed for 10 min at
70°C, and the reaction was incubated for 1 h at 42°C,
followed by enzyme inactivation for 10 min at 90°C.

20

    PCR

        DNA fragments of SPRY4A, MAFbx, FZD8, PDE2A,
RTP801, FLJ20898, ARHGEF2, and β-actin were amplified
by PCR using cDNAs obtained by reverse transcription of
25 mRNA from GIST cells and tumor biopsies as a template and
the following sets of primers: Sprouty 4A (SPRY4A), 5'-
CCGTTCTGTGGAGAGTCGATTTAC-3' (SEQ ID NO: 1) and 5'-
GTCCCTCAGTGGCTCTCGACT-3' (SEQ ID NO: 2); Frizzled 8
(FZD8), 5'-ACAGTGTTGATTGCTATTAGCATG-3' (SEQ ID NO: 7) and
30 5'-GTGAAATCTGTGTATCTGACTGC-3' (SEQ ID NO: 8); ARHGEF2,
5'-AAGGACGGAGAAAGGGAGAA-3' (SEQ ID NO: 9) and 5'-
CAAGACAGCAGTGACCCTGA-3' (SEQ ID NO: 10); PDE2A, 5'-
CCGCGATCTTTCTCGTAGTC-3' (SEQ ID NO: 11) and 5'-

CCCACTTCTGCTACCTGCTC-3' (SEQ ID NO: 12); MAFbx, 5'-

GTCCTGGGGTGAAAGTGAAA-3' (SEQ ID NO: 13) and 5'-

TCACAGCTCACATCCCTGAG-3' (SEQ ID NO: 14); FLJ20898, 5'-

CCCGAGTGACTCTGTTTTCC-3' (SEQ ID NO: 15) and 5'-

5   ACACCCAGTTGGAGGTGAAG-3' (SEQ ID NO: 16); RTP801, 5'-

AGACACGGCTTACCTGGATG-3' (SEQ ID NO: 17) and 5'-

TTGATGACTCGGAAGCCAGT-3' (SEQ ID NO: 18); and β-actin, 5'-

CTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 19) and 5'-

CATGATGGAGTTGAAGGTAGTTTCGT-3' (SEQ ID NO: 20). PCR was

10   performed in a reaction volume of 30 μl containing cDNA

from 1 μl of the reverse transcription reaction described

above as a template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

1.5 mM MgCl$_2$, 0.001% gelatin, 0.5 μM of both the forward

and reverse primer, 60 μM of each deoxyribonucleotide,

15   5% DMSO, and 0.5 unit of platinum Taq DNA polymerase

(Invitrogen). After an initial denaturation step at 95°C

for 5 min, DNA was amplified through 19, 21, 22, 23, 24,

25, and 27 cycles consisting of 5-s denaturing at 95°C,

60-s annealing at 55°C, and 90-s extension at 72°C. The

20   products were resolved on a 1.5% agarose gel and

visualized by UV light after staining with ethidium

bromide. Images of the gels were obtained using an Alpha

Imager 2200 documentation and analysis system (Alpha

Innotech, San Leandro, CA). Images were captured within

25   linear dynamic range and controlled for white color

saturation. DNA bands were quantified using the Alpha

Imager version 5.5 software package and the Fuji Image

Gauge version 3.11 software package (Fuji Photo Film Co.,

Ltd.).

30

Results:

To further elucidate the role of the members of the

SPRTY family, RT-PCR was performed on SPRY1, 2, 3, and 4

in untreated and treated imatinib GIST cells. As seen in
Figure 7A, SPRY1, 2, and 3 mRNA levels are relatively low
and increased following treatment with imatinib. This is
in contrast to the loss of expression of SPRY4.

5   Additionally, a Western blot was performed on lysates
from GIST cells that had been treated with imatinib for
24, 72, and 120 hours (Fig. 7B). Notably, the protein is
nearly absent by 24 hours and is essentially undetectable
through the 120 hour timepoint.

10   To further support the array data, a panel of cDNA
probes and a set of PCR primers for each of the named
genes were employed in Northern and RT-PCR analyses,
respectively, using an aliquot of RNA reserved from the
array experiments (Fig. 8A; data not shown). The

15   saturation of the PCR reaction was controlled by sampling
each reaction together with a β-actin control reaction at
different numbers of PCR cycles. All seven genes were
differently expressed, as expected from our microarray
screen (Fig. 8). SPRY4A, ARHGEF2 (Rho/Rac guanine

20   exchange factor), RTP801, FLJ20898, and FZD8 (Frizzled
Homlog 8) mRNA levels were diminished significantly
within 6 h after addition of imatinib, and the level of
PDE2A (phosphodiesterase 2A) mRNA decreased measurably 12
h after drug treatment. In contrast, MAFbx levels were

25   dramatically induced within 6 h of imatinib exposure and
remained elevated throughout the treatment (Fig. 8A).

     The panel of imatinib-responsive genes was also
tested for dependency on ERK1/2 activation by MEK1.
Expression patterns of SPRY4A, MAFbx, and ARHGEF2 in

30   U0126 treated cells were similar to those after imatinib
treatment (Figs. 8B and 8C). PDE2A and RTP801 mRNA
levels were decreased transiently, and the levels of
FLJ20898 and FZD8 did not appear to change noticeably

(Fig. 8B). The level of expression after addition of
U0126 was comparable with that seen with imatinib, except
that the duration of suppression with a single dose of
imatinib was more prolonged (data not shown).

5   The effect of LY294002, a PI3K inhibitor (Vlahos,
C.J., et al. (1994) J. Biol. Chem. 269:5214-5248) was
also evaluated. Little or no effect on expression levels
was observed with any of the imatinib-responsive genes in
the restricted panel (Fig. 5D; data not shown). These

10 results suggest that several of these genes are likely to
be dependent on activation of the MAPK signaling pathway
by c-KIT, whereas others may not be directly downstream
of either MAPK or AKT signaling pathways.

       Additionally, the effects of imatinib on the GRB-
15 associated docking proteins (GAB) were studied. GAB has
been shown to be an adaptor protein capable of binding to
many different RTKs (e.g., EGFR, insulin receptor, and
cytokine receptor) and other signaling proteins (e.g.,
GRB2, phospholipase Cg, SHP2, PIK3, Shc, and Crk; see,

20 for example, Holgado-Madruga, M., et al. (1996) Nature
379:560-564; Lehr, S., et al. (1999) J. Biol. Chem.
276:12257-12265). Western blot analysis of lysates from
GIST882 cells treated with imatinib demonstrated that
while the protein levels of GAB1 remained steady through

25 the treatment, the amount of phosphorylated GAB1
decreased significantly after 30 minutes (Fig. 9A).
Notably, GAB2 protein levels and phosphorylation state
did not change with treatment (Fig. 9B). These data
reveal that the efficacy of imatinib treatment may also

30 be correlated with alterations in post-translational
modification of different target proteins.

While certain embodiments of the present invention have been described and/or specifically exemplified above, various other embodiments will be apparent to those skilled in the art from the foregoing disclosure.

5 The present invention is, therefore, not limited to such embodiments, but is capable of considerable variation and modification without departing from the scope of the following claims.